MONOCLONAL ANTIBODY

This invention relates to monoclonal antibodies specific for the receptor for human granulocyte-macrophage colony stimulating factor (hGM-CSF), and to compositions and methods utilizing these antibodies.

Background and Prior Art

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a growth and differentiation factor for a variety of haemopoietic progenitor cells (including those for neutrophils, macrophages, eosinophils, megakaryocytes and erythroid cells) and can also functionally activate mature neutrophils, eosinophils and macrophages (1-4). All of the actions of GM-CSF are thought to be mediated through the interaction of GM-CSF with specific cell surface receptors. These receptors consist of a specific α-chain (PCT/AU90/00342, published as WO 91/02063) (5), which binds GM-CSF with low-affinity, and a common β-chain, which by itself does not bind GM-CSF with detectable affinity, and is shared by the α-chains for the interleukin-3 and interleukin-5 receptors (6,7). The α-β complex generates high-affinity binding sites for GM-CSF, and is required for cell signalling (8). However, the mechanism of conversion of the low affinity receptor to high affinity is not yet understood.

Since murine models of excess GM-CSF production (9,10) have indicated that a variety of disease states can result from over-production of GM-CSF and consequent macrophage accumulation, and since clinical trials of GM-CSF have indicated that some disease states can be exacerbated by the action of GM-CSF (11), we believe that antibodies that inhibit GM-CSF action may have clinical

utility. Moreover, since several human myeloid leukaemias have been shown both to possess GM-CSF receptors and to respond to GM-CSF by proliferation (12-16), we believe that such antibodies could be used to suppress leukaemic cell proliferation or to target leukaemic cells for antibody-mediated killing. Finally we believe that identification of the antibody epitope on the GM-CSF receptor α-chain recognized by neutralizing antibody will aid in the design of selective peptide and non-peptide antagonists of GM-CSF action.

We have generated a panel of monoclonal antibodies that specifically recognize the human GM-CSF receptor α-chain, and one of these inhibits the capacity of GM-CSF to bind to its cellular receptor and to biologically stimulate cells that bear GM-CSF receptors. Surprisingly, this inhibition occurs on cells that bear either the α-chain of the receptor alone (FD-A5 cells), or which bear the high-affinity α-β complex (AML 193 cells, human bone marrow cells).

Summary of the Invention

According to a first aspect, the invention provides monoclonal antibodies specific for hGM-CSF receptor, which antibodies have the ability to inhibit the biological activity of hGM-CSF. Preferably the monoclonal antibodies of the invention are able to bind to both the high affinity and to the low-affinity GM-CSF receptor.

In a preferred embodiment, the monoclonal antibody is KI-2B7-17-A.

According to a second aspect, the invention provides hybridoma cell lines producing these monoclonal antibodies.

According to a third aspect, the invention provides therapeutic compositions comprising a monoclonal antibody according to the invention, together with a pharmaceutically acceptable carrier.

According to a fourth aspect of the invention, there is provided a method of treatment of a GM-CSF dependent condition, comprising the step of administering to a patient in need of such treatment an effective amount of a monoclonal antibody according to the invention, optionally together with a pharmaceutically-acceptable carrier. It is contemplated that methods of treatment either by suppression of leukaemic cell proliferation, or by targeting of leukaemic cells for antibody-mediated killing are within the scope of the invention. This method of the invention is applicable to both acute and chronic myeloid leukaemia, and to other leukaemias which are influenced by GM-CSF.

In a particularly preferred embodiment, the method of the invention provides a method of mitigation of the side-effects of treatment with GM-CSF, comprising the step of administering to a patient in need of such treatment an effective amount of a monoclonal antibody of the invention, optionally together with a pharmaceutically-acceptable carrier.

In a further aspect, the invention provides a method of screening of selective antagonists to GM-CSF, comprising the step of utilizing an antibody of the invention to identify the specific epitope on GM-CSF receptor recognised by said antibody, and testing putative antagonists of GM-CSF action for ability to bind to said epitope. The antagonists may be either peptides, or non-peptide molecules.

Using the methods described herein, screening of a relatively small number of hybridoma clones revealed 15

candidate clones, of which one surprisingly produced a monoclonal antibody having inhibitory activity against high-affinity hGM-CSF receptor. Thus hybridoma clones having the required specific activity can be produced at high frequency, and it is considered that the skilled person would readily be able to utilise the methods described herein in order to produce neutralising monoclonal antibodies.

Similarly, methods for producing "humanised" forms of monoclonal antibodies, or for producing antigen-binding fragments of monoclonal antibodies, by the use of genetic engineering techniques are well-known in the art. Thus such humanised forms or antigen-binding fragments are also to be clearly understood to be within the scope of the invention.

Detailed Description of the Invention

The invention will now be described by way of reference only to the following non-limiting examples, and to the accompanying drawings, in which

Figure 1 shows the results of immunodepletion assays in which the ability of monoclonal antibodies to deplete solubilized hGM-CSF receptor α-chain from solution was tested;

Figure 2 summarises results of immunofluroescence detection of hGM-CSF receptor α-chain on FDA5 cells;

Figure 3 shows results of direct binding inhibition studies, showing dose-dependent inhibition by a representative antibody of the invention of binding of GM-CSF to receptor;

Figure 4 shows suppression of specific binding of GM-CSF to receptors by the antibody of the invention;

Figure 5 shows that only one of the five monoclonal antibodies tested had the ability to directly

inhibit binding of hGM-CSF to solubilised receptor;

Figure 6a shows results of binding studies to determine the binding affinity of an antibody of the invention to hGM-CSF receptor;

Figure 6b shows corresponding Scatchard plots;

Figure 7 shows the kinetic association and dissociation rates of binding of an antibody of the invention to hGM-CSF receptor.

Figure 8 shows inhibition by an antibody of the invention of colony formation by human bone marrow cells in response to hGM-CSF;

Figure 9 shows that this inhibition is dose-dependent;

Figure 10 illustrates the ability of an antibody of the invention to inhibit growth *in vitro* of an hGM-CSF dependent cell line; and

Figure 11 shows inhibition of stimulation by hGM-CSF of a cell line transfected with hGM-CSF receptor α-chain.

In the following examples, the invention is described in detail with reference to monoclonal antibody produced by the hybridoma cell line KI-2B7-17-A. A sample of this hybridoma cell line was deposited under the Budapest Treaty in the Public Health Laboratory Service Centre for Applied Microbiology and Research European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire, SP4 0JG, U.K. on 23 September 1992, under Accession Number 92092317.

Abbreviations used herein are as follows:

DME	Dulbecco's modified Eagle's medium
FCS	foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor

ICAM-1 Intercellular adhesion molecule-1

SAMIg Sheep anti-mouse immunoglobulin

All of the cell lines referred to herein are publicly available, or have been previously described in the literature.

Example 1 Hybridoma Production

In initial studies, mouse FDC-P1 cells transfected with the human GM-CSF receptor α-chain (FDA5 cells) (17) were injected into syngeneic BALB/c recipients, in the expectation that this would result in more specific immune responses. No successful fusions resulted with this protocol. Since our prior observations had shown that allogeneic responses enhanced the immune response to other cell surface antigens, we immunized CBA/J mice with allogenic FDA5 cells. The mice received weekly intraperitoneal injections of $2\text{-}3\times10^7$ viable FDA5 cells for four weeks, and sera were assayed in the immunodepletion assay (see below). The mice were then rested for at least four weeks before a final immunization. Four days after this final boost the animals were killed by cervical dislocation and the spleens removed. Single cell suspensions were prepared and washed in serum-free Dulbecco's modified Eagle's medium (DME). The spleen cells were fused with NS-1 myeloma cells in the presence of 50% (w/v) polyethylene glycol 4000 (Boehringer-Mannheim) (18).

The cells were gently resuspended in DME/15% (v/v) foetal calf serum (FCS) 10% (v/v) conditioned medium from the P33D18 macrophage cell line (as a source of interleukin-6), and cultured in 96 well Falcon microwell flat bottom trays at 2×10^5 cells per well. The cells were fed with a standard selective medium containing hypoxanthine, aminopterin and thymidine (HAT medium) after overnight culture, and thereafter every three days. After

7-14 days, developing hybridoma clones were observed in the wells and supernatants were harvested for testing.

Example 2 Screening of Hybridoma Clones

a) Analysis of COS 7 Cell Transfectants by Immunofluorescence.

COS 7 cells (ATCC CRL 9589) were electroporated with expression plasmids encoding the α-chain of the human GM-CSF receptor (5) or the cell surface molecule ICAM-1 (19). After three days the cells were harvested, washed and analyzed with candidate hybridoma supernatants by indirect immunofluorescence. Briefly, 10^6 cells were incubated with 50 µl of culture supernatant for 30 min at 4°C. The cells were washed, resuspended in an appropriate dilution of FITC-$(Fab_2)'$ fragments of sheep anti-mouse immunoglobulin antibody (FITC SAMIg) (DDAF, Silenus), and held on ice for 30 min at 4°C. The cells were washed and then fixed in 1% formalin in phosphate-buffered saline (PBS). The samples were examined by fluorescence microscopy and 35 putative positives (GMR^+, $ICAM^-$) identified from a total of 390 wells. The cells from these wells were expanded to generate further supernatants for testing and to provide cells for recloning and cryopreservation. Fifteen clones remained positive by immunofluorescence and were further selected by screening in immunodepletion assays (see below).

(b) Immunodepletion Assays.

COS 7 cells that had been electroporated with the human GM-CSF receptor α-chain cDNA in the CDM8 vector 72 hours previously were lysed, and membranes purified as previously described (20). The membranes were extracted with 1% Triton X-100, centrifuged at 200,000g for 15 min

and the supernatant retained as solubilized GM-CSF receptor α-chain.

Solubilized GM-CSF receptor α-chain (50 µl) was preincubated with hybridoma supernatant (100 µl), sheep anti-mouse immunoglobulin (100 µl of a 1/100 dilution) and protein-G Sepharose (100 µl of a 1/5 suspension) in a final volume of 400µl of Hepes-buffered (20 mM, pH 7.4) RPMI medium containing 10% (v/v) foetal calf serum for 60 min at 23°C with rotation. Tubes were then centrifuged at 14,000 g for 10 sec and the supernatants recovered.

Supernatants (50 µl aliquots) were incubated with ^{125}I-human GM-CSF (10 µl, ~200,000 cpm, specific radioactivity 30,000 cpm/ng) in duplicate, with or without unlabelled human GM-CSF (10 µl of 50 µg/ml) and concanavalin A-Sepharose 4B (30 µl of a 1/4 suspension in 0.1M sodium acetate buffer pH 6.0), for 60 min at 23°C with rotation. The incubation mixture was then layered over 180 µl of foetal calf serum in small centrifuge tubes, centrifuged at 14,000 g for 10 sec and the pellet removed by cutting the tube with a scalpel blade. Specific binding in these pellets was calculated as the binding (cpm) without unlabelled GM-CSF minus the binding in the presence of unlabelled GM-CSF.

The presence of monoclonal antibody against the human GM-CSF receptor α-chain was detected by the ability of the appropriate hybridoma supernatant to deplete soluble human GM-CSF receptor α-chain in the preincubation step (relative to control hybridoma supernatant or antibody), as revealed by a reduction in the specific binding of ^{125}I-human GM-CSF detected in the subsequent incubation.

Example 3 Direct Binding Inhibition Assays.

Solubilized human GM-CSF receptor α-chain (50 µl), monoclonal antibody or hybridoma supernatant (10 µl), ^{125}I-human GM-CSF (10 µl, ~200,000 cpm), and Concanavalin A-Sepharose 4B (30 µl of a 1/4 suspension) with or without unlabelled human GM-CSF (10 µl of 50 µl/ml), in a total volume of 110 µl of 0.1M NaAcetate buffer pH 6.0 were incubated at 23°C for 60 min with rotation, and specific binding was determined as described above.

Example 4 Isotype Determination of Monoclonal Antibodies

Supernatants from positive hybridoma clones were isotyped using the Amersham Isotyping Kit (Amersham, Buckinghamshire, England). Monoclonal antibody KI-2B7-17A, along with several other monoclonals, was found to be of the IgG2a type.

Example 5 Purification of Monoclonal Antibodies

Individual hybridoma cell lines were expanded to 10 litres of culture volume and the supernatants harvested and concentrated 16-fold. The concentrated supernatants were loaded on to a protein-A-Sepharose CL-4B column (5 ml) (Pharmacia, Uppsala, Sweden), and the column was washed extensively with phosphate (20 mM, pH 7.4) buffered 0.15M saline (PBS) until the absorbance at 280 nm was negligible. The column was then eluted with 0.1M glycine HCl buffer pH 3.0 and fractions collected into 1/10 volume of 1.0M Tris HCl pH 8. The eluates were concentrated and dialyzed extensively against PBS before use.

Example 6 Radiolabeling of Monoclonal Antibodies

Monoclonal antibodies were radioiodinated with ^{125}I either by the modified iodine monochloride method described previously (21), or by the use of ^{125}I-Bolton-Hunter reagent (22). Monoclonal antibody KI-2B7-17-A only retained binding activity when iodinated using the Bolton-Hunter reagent, so direct binding studies were performed with this form of the iodinated antibody (specific radioactivity 50,000 cpm/ng).

Example 7 Immunodepletion Studies

Figure 1 shows the capacity of randomly selected hybridoma supernatants to deplete solubilized human GM-CSF receptor α-chain from solution. After immunodepletion, the solution was tested for residual specific binding of ^{125}I-human GM-CSF. Mean specific cpm bound and standard deviation are shown by the horizontal line and error bar in Figure 1 for solutions that had not been immunodepleted. Most hybridoma supernatants did not reduce the specific binding, but supernatants 37, 38 and 56 clearly caused a marked reduction in specific binding, indicating that they contained antibodies that could recognize the human GM-CSF receptor α-chain. In addition the serum from immunized mouse number 8 also contained antibodies which recognized the receptor. This assay allowed the identification of five purified monoclonal antibodies (KI-3G9-25-1, KI-3G3-14-15, KI-2B7-17-A, K3-2E9-4-1 and K3-2C7-16-1), each derived from double cloned hybridoma cell lines that recognized the human GM-CSF receptor α-chain.

Example 8 Immunological Detection of Human GM-CSF Receptor α-chain on FDA5 Cells

Figure 2 shows that FDA5 cells (17), but not parental FDC-P1 (FD) cells (17), showed significant fluorescence above background when stained with the monoclonal antibodies KI-2B7-17-A and FITC SAMIg and then subjected to flow cytometry (solid line, unfilled). The positive control, indicated in black, shows fluorescence with anti-H2D antibodies. The dotted profile represents fluorescence of cells treated with FITC-SAMIg in the absence of test antibody.

Example 9 Direct Binding Inhibition Studies

Figure 3 shows that when monoclonal antibody KI-2B7-17-A was co-incubated with solubilized human GM-CSF receptor α-chain and ^{125}I-human GM-CSF (with no prior immunodepletion step), the antibody directly interfered with ^{125}I-GM-CSF binding to the receptor, as evidenced by the dose-dependent inhibition of the amount of ^{125}I-GM-CSF bound to the receptor which was observed.

This antibody also completely suppressed specific binding of ^{125}I-GM-CSF to GM-CSF receptors on cells of the HL60 promyelocytic leukaemic cell line, which had been induced to differentiate by exposure to 1% dimethylsulphoxide for 5 days, as shown in Figure 4.

When KI-2B7-17-A antibody (10μg/ml) was co-incubated with the cells and ^{125}I-human GM-CSF (0-10^6 cpm, 0-3 ng/ml) it reduced the specific binding (filled squares) to the same extent as unlabelled human GM-CSF (5 μg/ml) (open circle).

Figure 5 shows that monoclonal antibody KI-2B7-17-A was unique amongst the five purified monoclonal antibodies directed against the human GM-CSF receptor α-

chain, in that it directly inhibited the binding of ^{125}I-human GM-CSF to solubilized receptor. This suggested that KI-2B7-17-A recognized a unique epitope on the GM-CSF receptor α-chain which is involved in GM-CSF binding. This was confirmed by showing that only unlabelled KI-2B7-17-A, and not the other four monoclonal antibodies, could compete for the direct binding of ^{125}I-KI-2B7-17-A to human GM-CSF receptor α-chain expressed on COS cells (Figure 5).

Example 10 Direct Binding of KI-2B7-17-A to Human GM-CSF Receptor

In order to demonstrate the direct binding of the KI-2B7-17-A antibody to human GM-CSF receptor and determine its binding affinity, the antibody was iodinated using ^{125}I-Bolton-Hunter reagent. Increasing amounts of ^{125}I-KI-2B7-17-A were incubated with U937 human monocytic cells (ATCC CRL 1593) as a source of natural high-affinity human GM-CSF receptor, or with COS cells transfected with human GM-CSF receptor α-chain, and specific binding was determined as the difference in cpm bound in the presence or absence of unlabeled KI-2B7-17-A (12 µg/ml). Figure 6a shows the binding isotherms generated at 4°C, and their transformation into Scatchard plots (20) is shown in Figure 6b. The binding affinity of the antibody to U937 cell GM-CSF receptor was characterized by an equilibrium dissociation constant (K_D) of 1.35 nM and 2500 sites/cell. This compares to a K_D of 0.41 nM and site number of 1500 sites/cell for ^{125}I-human GM-CSF on the same cells (data not shown). A similar affinity of binding of the antibody to transfected COS cells was determined (K_D=3.15 nM), although a much higher number of receptors was detected (490,000/cell). The solid line in panel B of Figure 6b is the same as that in panel A, drawn to the same scale for

comparative purposes. With these affinity constants KI-2B7-17-A should give 50% inhibition at between 0.1-1 µg/ml, and this accords with the observations in example 11.

We further characterized the direct binding of ^{125}I-KI-2B7-17-A antibody to natural human GM-CSF receptor on U937 cells by measuring its rate of association (k_{on}) and rate of dissociation (k_{off}) from the receptor (Figure 7). In Part A of this figure, U937 cells ($2.2x10^6$ per point) were incubated with ^{125}I-KI-2B7-17-A antibody (500,000 cpm/50 µl/point) for at least 1 hr at 4°C. The cells were then rapidly washed and resuspended in binding medium containing 20 µg/ml of unlabeled antibody. At the indicated time points aliquots of cells were removed, and the specifically bound radioactivity remaining determined as described for other figures. k_{off} was determined from the plot in Figure 7A as 0.002 min^{-1} (k_{off}=ln 2/t½). In Part B the same number of U937 cells and ^{125}I-KI-2B7-17-A antibody were mixed, and aliquots removed at the indicated times at 4°C to determine specifically bound radioactivity (B_t). The data were extrapolated to determine the amount bound at infinite time (Be), and the data plotted as ln[Be/(Be-Bt)] versus time. The slope of the line is k_{on}[Antibody]+k_{off}, so since the concentration of ^{125}I-KI-2B7-17-A was known, k_{on} could be calculated as $3.1x10^6$ M^{-1} min^{-1}. The slow dissociation rate of the antibody (0.002 min^{-1}) makes it useful as an inhibitor of the GM-CSF receptor.

Eample 11 Inhibition of the Biological Action of GM-CSF by MonoclonalAntibody KI-2B7-17-A

Monoclonal antibody KI-2B7-17-A (filled squares), but not the isotype matched monoclonal antibody KI-3G3-14-15 (open circles), which was obtained from a similar fusion

and also recognizes the human GM-CSF receptor α-chain, nor saline (closed circles), was able to inhibit colony formation from human bone marrow cells stimulated by human GM-CSF in semisolid agar cultures *in vitro* (Figure 8). At 10 µg/ml, KI-2B7-17-A almost completely inhibited colony formation induced by up to 1500 U/ml of GM-CSF when cultures were scored at day 7. A similar but less marked inhibition was seen when cultures were scored at day 14. Figure 9 shows that the inhibition of colony formation (stimulated by 400 U/ml of GM-CSF) was dependent on the dose of antibody KI-2B7-17-A, with 50% inhibition occurring at about 1 µg/ml and nearly complete inhibition occurring at about 10 µg/ml.

Figure 10 shows that antibody KI-2B7-17-A also inhibited the growth *in vitro* of the human GM-CSF-dependent acute monocytic leukaemia cell line AML-193 (ATCC CRL 9589), as assessed by the inhibition of incorporation of ^{3}H-methyl-thymidine into cellular DNA. 50% inhibition again occurred at 1 µg/ml of antibody, and nearly complete inhibition of GM-CSF-stimulated growth occurred at 10-100 µg/ml. The solid line with a filled square and error bars represents incorporation into cells in the absence of added antibody, while the solid line with open square and error bars represents incorporation in the absence of GM-CSF. No signficant inhibition was observed with the isotype-matched antibody KI-3G9-2S1 (closed circles)

Finally Figure 11 shows that antibody KI-2B7-17-A inhibited the growth *in vitro* in semisolid agar cultures of the FD-A5 cells (mouse FDC-P1 cells transfected with human GM-CSF receptor α-chain) stimulated by human GM-CSF, as assessed by colony formation and colony size. Again 50% inhibition occurred at about 1 µg/ml, and nearly complete inhibition occurred by 10 µg/ml. In the figure only the

excess colonies stimulated above the saline control by GM-CSF are shown, with saline addition indicated by closed circles, KI-3G3-14-15 antibody by open circles, and KI-2B7-17-A antibody by closed triangles.

References cited herein are listed on the following pages.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

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CLAIMS

1. A monoclonal antibody specific for human GM-CSF receptor (hGM-CSF receptor), said antibody having the ability to inhibit the biological activity of hGM-CSF, and said antibody being able to bind to both the high affinity and to the low-affinity GM-CSF receptor.

2. A monoclonal antibody according to Claim 1, said antibody having the ability to inhibit the binding of GM-CSF to the high-affinity hGM-CSF receptor.

3. Monoclonal antibody KI-2B7-17-A.

4. A fragment or derivative of a monoclonal antibody according to any one of Claims 1 to 3, having the ability to inhibit the biological activity of GM-CSF.

5. A monoclonal antibody according to any one of Claims 1 to 4, or a biologically active fragment or derivative thereof, produced by genetic engineering methods.

6. A therapeutic composition comprising a monoclonal antibody according to any one of Claims 1 to 3, or a fragment or derivative according to Claim 4 or Claim 5.

7. A hybridoma cell line producing an antibody according to any one of Claims 1 to 5.

8. Hybridoma cell line KI-2B7-17-A, as deposited in the European Collection of Animal Cell Cultures on 23 September 1992 under Accession Number 92092317.

9. A method of treatment of a GM-CSF dependent condition, comprising the step of administering to a patient in need of such treatment an effective amount of an antibody, antibody fragment or antibody derivative according to any one of Claims 1 to 5, optionally together with a pharmaceutically-acceptable carrier.

10. A method of treatment according to Claim 9, wherein the disease to be treated is selected from acute or chronic myeloid leukaemia, or other leukaemias which are influenced by GM-CSF.

11. A method of mitigation of the side-effects of treatment with GM-CSF, comprising the step of administering

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to a patient in need of such treatment an effective amount of an antibody, antibody fragment or antibody derivative according to any one of Claims 1 to 5, optionally together with a pharmaceutically-acceptable carrier.

12. A method of screening of selective antagonists to GM-CSF, comprising the step of utilising an antibody, antibody fragment or antibody derivative according to any one of Claims 1 to 5 to identify the specific epitope on the GM-CSF receptor recognised by said antibody or antibody derivative, and testing putative antagonists of GM-CSF action for the ability to bind to said epitope and inhibit the biological activity of GM-CSF.

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